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THE DETERMINATION OF THE NUMBER OF BODY CELLS IN MILK BY A DIRECT METHOD.*

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FOR some time sanitarians have felt that it was important to be able to determine the number of body cells[1] in milk. Large numbers have been held to be undesirable inasmuch as such conditions seem to be associated with abnormal conditions of the udder.

At the present time, the method in most general use in Board of Health and similar laboratories for determining the number of these body cells present in a given sample of milk is the so-called "smeared sediment" method first devised by Stokes and Wegefarth[2] of Baltimore. Later this method was modified by Stewart[3] of Philadelphia and Slack[4] of Boston. These modifications have improved the accuracy of the method but the results obtained with it have not been entirely satisfactory.

In 1905, a "volumetric" method for determining the number of body cells was suggested by Doane and Buckley.[5] This method uses the well-known Thoma-Zeiss hemacytometer for examining the sediment secured by the use of the centrifuge. Practically the same method was devised independently by Savage.[6] The results obtained by this method show larger numbers of body cells present than do those obtained by the "smeared sediment" method. Nearly all of the research work along this line is being done by the "volumetric" method because there seems to be general agreement that it is more accurate than the "smeared sediment" method. Russell and Hoffman[7] find that they get better

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[1] On account of the recent work claiming that the cellular elements present in milk are not leukocytes but detached epithelial cells, the noncommittal term "body cells" will be used throughout this paper instead of the more common expressions "leukocytes" or "pus" cells.

[2] *Med. News*, 1897, 71, p. 45.

[3] *Amer. Med.*, 1905, 9, p. 486.

[4] *Jour. Infect. Dis.*, 1906, Supplement No. 2, p. 214.

[5] *Md. Agri. Exper. Sta. Bull.*, 1905, 102, p. 205.

[6] *Jour. Hyg.*, 1906, 6, p. 123.

[7] *Wis. Agri. Exper. Sta. 24th Ann. Rep.*, 1907, p. 231.

results by heating their samples before centrifuging, and their recent work has been done in this way.

A method whereby it is possible to determine the amount of sediment present in milk and indirectly the number of body cells has been proposed by Trommsdorff.[1] This uses the well-known principle of the hematocrit. It has not found general favor in this country.

THE NEW METHOD.

During the summer of 1909, an entirely new method for determining the number of body cells present in milk was devised in the Boston Bio-Chemical Laboratory. By this method, the number of cells present is determined by a direct examination of the milk without recourse to the centrifuge, thereby simplifying the operation and also avoiding the very serious error which must be present in any method based on the use of the centrifuge. Without going into the details of the steps which led up to the devising of this method, it may be described as follows:

The sample is well shaken to distribute the cream equally through the milk. A measured drop (0.01 c.c.) is then withdrawn by means of a specially constructed capillary pipette with a rubber bulb.[2] This drop is then spread evenly over an area of 1 sq. cm. on an ordinary glass slide. The milk is then dried with gentle heat, the fat dissolved out with xylol or other fat solvent, the smear fixt to the slide by immersion in alcohol for a few minutes, dried, overstained with methylene blue, and decolorized with alcohol. The slide is then ready for examination.

When well done the smear presents an even appearance and the cells show up clearly on a bluish field. This background is the dried casein and other milk solids, and shows holes where the fat drops have been removed. Some difficulty was experienced at first in getting the smears to stick to the slide, but since the adoption of the method of treating with alcohol before staining there has been no trouble of this sort. The cells are evenly distributed even at the edges where there is frequently a somewhat

[1] *Berlin Tierarztl. Wchnschr.*, 1906, p. 81.

[2] May be obtained of Bausch and Lomb Optical Co., Rochester, N.Y., at a cost of fifty cents each.

thicker border of milk solids. Rapid drying is necessary in order to prevent the segregation of the fat drops, a thing which tends to make the distribution of the cells uneven.

Where large numbers of examinations are to be made, it will be found convenient to use the 2×8 inch slides now in use in many laboratories using the "smeared sediment" method. A convenient way to determine the 1 sq. cm. area is to place the slide to be used over one of the ruled plates used in counting bacterial colonies. It will be found somewhat difficult to get even smears in all cases, so that smears should always be made in duplicate.

The determination of the number of cells present is made with the microscope, using the oil-immersion lens. If the diameter of the field is so arranged that it equals 0.16 mm., then each field covers approximately 0.005 of a square centimeter. This adjustment may be made by the use of the draw tube of the microscope. On this basis each cell seen in a field taken at random represents 500,000 cells per c.c. If 100 fields are counted and the total number of cells seen be obtained, then each cell represents 5,000 cells per c.c. This reduces the probable error to a smaller amount than would be necessary for routine work. If the smears are well prepared, the cells may be readily seen and counted with lower powers of the microscope, thus making the counting less laborious.

A series of 31 tests done in duplicate where 100 fields of the oil-immersion lens were counted on each smear shows a variation of 14.5 per cent. In this series of tests, there are two cases with an error greater than 30 per cent (i.e., 42.9 per cent and 64.3 per cent), but both of these were cases where the cellular content was less than 250,000 per c.c. In such cases, the number of cells present in the smears is so few that an error of 50 per cent or more is not surprising, and is of little practical consequence.

The error seems to be due to inaccuracy of count rather than in the preparation of the smear. Even on the same smear a difference of 15 per cent may occur in two different counts. The error is materially lessened if the test is done in duplicate, 100 fields counted on each smear, and the results averaged. All of the results given in this paper were obtained in this way.

DISTRIBUTION OF THE BODY CELLS IN CENTRIFUGED SAMPLES.

A careful examination of centrifuged samples of milk reveals the fact that the use of this instrument introduces so large and so variable an error that it practically vitiates the results obtained by both the "smeared sediment" and "volumetric" methods. It has usually been assumed, without sufficient ground for the assumption, that the centrifuge preciptates all but a small fraction of the cells and that whatever loss there is represents a fairly constant proportion of the whole. Savage made some investigation of the matter and reports that recentrifuging shows that approximately 12 per cent are lost and also that this fraction appears to be practically constant. He does not seem to have considered the possibility that he lost any large number of the cells by discarding the cream. Russell and Hoffman have followed up the question farther. They find that the number of cells precipitated in the sediment after centrifuging is increased by heating the milk to 60° C. They also state that they find more cells in the cream than in the skim milk where the cream was raised by gravity. Apparently this determination of the number of cells present in the cream was made by centrifuging the cream and determining the number of cells present by the "volumetric" method. If this was the case, the number of cells present must have been much larger than they observed, for, as we shall show, many would have been lost in the discarded cream.

An examination of centrifuged sample shows the following conditions:

Sample 1.—The milk used was taken from a quart bottle of market milk obtained from a first-class dealer. This dealer is one who has had the milk from all of his herds examined frequently for high cell content by the "smeared sediment" method. Where high numbers have been detected, the cows have been carefully examined by a veterinarian and the offending cow or cows removed from the herd.

a) The number of cells present was determined by the "direct" method by four tests as 1,440,000, 1,625,000, 1,240,000, and 1,260,000 per c.c. Average = 1,390,000 per c.c. or 2,780,000 per 2 c.c.

b) Seven 2 c.c. samples were centrifuged in the 2 c.c. rubber-stoppered tubes ordinarily used in the "smeared sediment" method (see Stewart) at 3,600 revolutions per minute for 10 minutes.

Numbers one to three of these tubes were used to make smears by the "smeared sediment" method. The smears were spread over an area of 4 sq. cm. and the total number of cells present in each smear was computed. Fifty to one hundred fields of the oil-immersion lens were counted in each case. No. 1 = 375,000, No. 2 = 165,000, No. 3 = 620,000. Average = 385,000. Some cells must have been lost by adhesion to the stopper, and so it is probable that the average number present in the sediment was 400,000, or approximately one-seventh of the actual number present.

A more thorough examination of the remaining tubes was made to determine the exact position of the remaining six-sevenths of the cells as accurately as possible. Previous experimentation had shown that a large proportion of the cells would be found in the cream, that a number would be present in the milk just under the cream and in the milk just above the sediment, and that the middle region would be comparatively free from the cells. The 2 c.c. tubes were therefore divided into regions as indicated in Fig. 1. In these A = the cream, B = the milk just below the cream (A + B = 1/2 c.c.), C = the middle region (1 c.c.), D = the lower 1/2 c.c., E = the sediment.

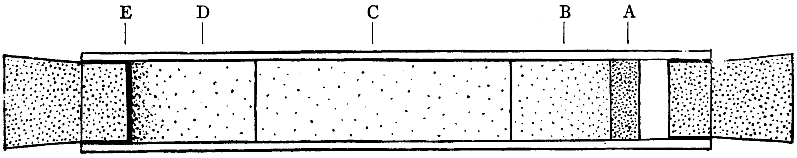

FIG. 1.—A drawing of a double-stoppered glass tube containing 2 c.c. of milk as it shows after being centrifuged. The part of the tube containing the milk is divided by vertical lines into Regions A, B, C, D, and E. A = the cream, B = the milk just below the cream (A + B = ½ c.c.), C = the middle c.c., D = the lower ½ c.c., and E = the sediment.

In Experiment 1, A contained 1,950,000 cells, B = 80,000, C = 50,000, D = 300,000, E = 400,000. Total = 2,780,000.

In Experiment 2, A = 1,330,000, B = 100,000, C = 55,000, D = 500,000, E = 625,000. Total = 2,610,000.

The number of cells present in the cream (A) could not be determined directly. However, an examination of the cream removed from tubes Nos. 1 and 2 was made by dissolving the cream in each case in 2 c.c. of ether in the rubber-stoppered tubes. The tubes were then recentrifuged. As a result of this treatment the cream appeared in the form of a nearly transparent jelly-like substance in the bottom of the tube. An examination of this jelly was made by drying some of it on a slide and then staining in methylene blue. It was impossible to make any accurate estimate of the number of cells present, but it was apparent that 1,000,000 or more were present.

The number of cells present in regions B and C was determined by the "direct" method in Tubes 4 and 5. Region B = 80,000 (average of 4 determinations). Region C = 50,000 (average of 12 determinations).

The number of cells present in Regions D and E in Tubes 4–7 was determined by a method which gives a good idea of the number actually present in the two regions combined, as some 20 previous tests on similar samples had shown. After all of the milk and cream had been removed from Regions A, B, and C, each tube was again filled with water and recentrifuged. This was done to reduce the amount of casein and other milk solids present in Region D by dilution and yet without appreciable loss of cells. All but 1/2 c.c. of the material in each tube was then discarded, the sediment thoroughly mixt with the remaining 1/2 c.c., and the whole spread evenly over an area of 4 sq. cm. on a glass slide and dried with gentle heat. After staining the smear with methylene blue, the total number of cells present was determined by the use of the oil-immersion lens in each of the four smears as follows: No. 4 = 620,000, No. 5 = 735,000, No. 6 = 715,000, No. 7 = 500,000. Average = 640,000. It has been arbitrarily assumed that enough cells were lost in the necessary manipulations to make the actual number 700,000. If the determination of the number of cells for the sediment (E) be taken as 400,000 as given above, then the number of cells for D is 300,000.

The numbers and distribution of the cells in the centrifuged samples are shown graphically in Fig. 1 already referred to. The number given for the cream (A) was obtained by subtraction from the total.

Sample 2.—A second sample of market milk obtained from an equally high-grade dealer was examined in a similar fashion.

a) Number of cells as determined by the "direct" method, four determinations: 1,350,000, 1,365,000, 1,280,000, 1,230,000. Average, 1,305,000 per c.c. or 2,610,000 per 2 c.c.

b) Two determinations were made by the "smeared sediment" method. 615,000 cells were found in the sediment of one 2 c.c. tube and 620,000 cells in the sediment of the other tube. Average, 617,000, or if loss due to manipulation be added, 625,000.

The average of four determinations made by the "direct" method showed the number of cells for Region B to be approximately 100,000.

The average of nine determinations by the same method showed 55,000 cells in Region C.

No direct determination of the number of cells present in Region D was made, but the number was estimated at 500,000. This estimate is unsatisfactory but is based on the result of some twenty or more similar examinations and may be regarded as approximately correct.

The number of cells present in the cream (A) was obtained by subtraction.

There are many imperfections in these determinations, and others are to be made, but the results are very suggestive. Moreover, the fact that this distribution of the cells in the milk after centrifuging corresponds with the known distribution of bacteria in similar samples as well as other data, indicates that these results may be relied upon. If so, it is clearly evident that no method of estimating the number of cells present, which is based upon the use of the centrifuge, can be relied upon to give even an approximate idea of the total number of cells present. The fact that the percentage of cream present in different samples of milk varies greatly, raises the question as to whether milks rich in butter fat would not show a relatively lower cell content than milks which are low in butter fat, if the "smeared sediment" or "volumetric" tests be used.

It will be noted that in one of these samples only one-seventh of the total number of cells present appeared in the sediment obtained by centrifuging, while in the other case nearly one-fourth appeared in the sediment. To test the question whether the ratio between the number of cells actually present and those obtained by centrifuging remained approximately constant, a series of 22 comparative tests were made using the "smeared sediment" and "direct" methods. The samples used were obtained from eight half-pint cans as they were delivered to the milk station from the farms supplying one of the high-grade dealers referred to above. Each sample is the mixt milk of several cows.

Smeared Sediment Method No. of Cells per c.c.	Direct Method No. of Cells per c.c.	Ratio
415,000	2,000,000	1: 4.8
40,000	640,000	1:16.0
75,000	430,000	1: 5.7
65,000	290,000	1: 4.5
95,000	330,000	1: 3.5
150,000	860,000	1: 5.8
245,000	850,000	1: 3.4
15,000	60,000	1: 4.0
115,000	310,000	1: 2.7
65,000	190,000	1: 3.0
1,650,000	10,690,000	1: 6.5
185,000	3,160,000	1:17.1
485,000	1,140,000	1: 2.4
230,000	610,000	1: 2.6
40,000	170,000	1: 4.3
90,000	3,710,000	1:41.2
340,000	2,830,000	1: 8.3
250,000	1,880,000	1: 7.5
1,060,000	2,310,000	1: 2.2
280,000	2,880,000	1:10.3
75,000	330,000	1: 4.4
775,000	1,540,000	1: 2
6,740,000	37,210,000	1: 5.2
Average = 305,000	1,690,000	

It will be seen from these tests that sometimes as many as one-half of the cells are present in the sediment while at other times not over one-fortieth of the total number are present. Under these circumstances, it is little wonder that the Committee on Standard Methods of Bacterial Milk Analysis appointed by the American Public Health Association recommends that little direct weight be given to the leukocyte tests now in use.

The fact that the number of cells present in the sediment can be materially increased by heating the milk to 60° C. before centrifuging as found by Russell and Hoffman is explained by these results. The heating changes the physical nature of the milk in such a way that more of the cells are precipitated and fewer go to the cream.

The fact that the results obtained by the "volumetric" method are higher than those obtained by the "smeared sediment" method is apparently due to the use of the milk just above the sediment as well as the sediment itself.

NORMAL NUMBER OF BODY CELLS PRESENT IN MILK.

A large series of determinations of the numbers of cells present in milk using this new method or some other direct method of examination must be made before it is possible to state the normal maximum. In addition to the 22 tests already given, 24 others were made of similar samples. In these as well as in the former case, the numbers given represent the average of two determinations in which 100 fields of the microscope were counted for each smear. No two of these samples came from the same farm, so that the combined results of the two tables give the conditions that obtain for 46 New England farms supplying milk to Boston. The samples were obtained during the month of August when the cattle were living on rations composed largely of grass supplemented by some grain or corn fodder.

NUMBER OF CELLS PER C.C. IN 24 SAMPLES.

90,000
1,660,000
230,000
80,000
810,000
690,000
540,000
900,000
3,620,000
1,430,000
2,010,000
1,580,000
1,580,000
820,000
750,000
1,410,000
210,000
1,100,000
660,000
2,330,000
3,610,000
1,500,000
170,000
3,380,000

Average of the 46 tests = 1,485,000 per c.c.

A series of examinations was made of quart bottles of market milk after they had been carried about the city of Boston on the regular morning delivery. It should be stated that seven of the eight samples were obtained from the same milk station and on the same morning. All of these samples were reported O.K. (i.e., showed less than 10 cells per field by the "smeared sediment" method) in the routine examination made in the laboratory. Samples obtained August 17 and 23, 1909:

1,310,000
3,414,000
5,210,000
2,290,000
1,390,000
4,480,000
2,170,000
2,510,000

Average of eight tests, 2,850,000 per c.c.

CONCLUSION AND SUMMARY.

1. The centrifuge precipitates between 2.5 per cent and 50 per cent of the total number of body cells present in milk. A greater percentage of these cells usually rises with the cream. The skim milk is practically free from them except just under the cream and just above the sediment.

2. The percentage of cells precipitated is so variable that no reliable estimate of the total number of cells present can be made from a determination of the number found in the sediment.

3. Therefore, no method based on the use of the centrifuge can be satisfactory.

4. A method of enumerating the number of cells in milk by an examination of smears of dried milk, made by using a definite quantity of milk and spreading it over a definite area, has been devised. The counting of the cells is done by means of the microscope after staining the smear.

5. A series of tests made by this method shows that the average number of these cells present in milk is approximately 1,500,000 per c.c. This number is far greater than has previously been supposed to be present, and the present standard (500,000) will need to be greatly increased if any numerical standard is retained. Very few samples contain less than 100,000 cells per c.c. One test of milk having a normal appearance and sold as market milk showed 10,690,000 cells per c.c.